

The University of Chicago

AN EXPERIMENTAL ANALYSIS OF THE
ALTMANN TECHNIC OF
FREEZING-DRYING

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF ANATOMY

1938

By

WILLIAM L. SIMPSON

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AN EXPERIMENTAL ANALYSIS OF THE ALTMANN TECHNIC OF FREEZING-DRYING¹

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ONE PLATE (EIGHT FIGURES)

The intelligent application of the Altmann technic of freezing-drying fixation to a specific cytological problem demands the evaluation and integration of the various factors in the method which might individually modify the ultimate quality of cellular preservation. The determination of factors that might affect the end result of the process had already begun at the time Gersh ('32) introduced the modern form of the technic. Gersh pointed out the effect on the final fixation of (1) the size of the sample of tissue frozen, (2) the relation of tissue volume to surface area, and (3) the water content of the tissue used. Scott ('33) noted (4) that the rate of freezing as determined by the heat conductivity of the freezing medium influenced the end result, as did (5) the temperature of dehydration. Hoerr ('36) in a review of the method added to these (6) the effects of changes in the rate of dehydration as influenced by the degree of vacuum employed and (7) the effect of miscellaneous factors involved in the handling of the dried material.

This paper presents results of an experimental evaluation of these and of other determinable factors for the role that each plays in adequate preservation of cellular constituents.

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MATERIALS AND METHODS

The results to be reported are based for the most part on guinea pig liver, pancreas, kidney, epididymis, duodenum, testis, adrenal, and spinal ganglion, and rabbit spinal cord and spinal ganglion. The pieces of test tissues ranged in size from half of a guinea pig thoracic dorsal root ganglion to whole guinea pig testes, kidneys, and spleens. To facilitate handling the individual pieces without losing track of them or of what had been done to them, the tissues were isolated in tiny numbered ballon silk bags. This did not appear to slow the process of dehydration.

In order to study the effect of increasing the ratio of surface area to tissue volume, tissues were stretched on cardboard, teased out, and even smeared on cover slips prior to freezing.

Freezing was brought about in (1) isopentane chilled in liquid nitrogen to temperatures ranging from -60 to $-195^{\circ}\text{C}.$, (2) directly in liquid nitrogen, (3) in liquid nitrogen after enclosing the test tissue in a thin covering layer of muscle or liver, and (4) on the surface of a brass block chilled in liquid nitrogen.

The latter method, freezing on a chilled brass block, was devised in an attempt (1) to obtain more rapid cooling of the tissues by the use of a substance having much higher capacity for heat conduction than either liquid nitrogen or isopentane, and (2) to establish definitely the direction of freezing through the tissue so that the progress of freezing might be correlated with the structural changes observed. The brass mounting block from a rotary microtome was used. Its concentric ridges made possible permanent identification of the side of the tissue with which it had been in contact.

To study the effect of freezing on the subsequent chemical fixation, tissues were frozen as usual and then thawed in chemical fixing solutions at temperatures of from 0° to $37^{\circ}\text{C}.$ Absolute alcohol, Zenker-formol, and a wide variety of the commonly employed fixatives were used in this manner. Dehydration, clearing, and embedding followed this procedure which will be referred to as "freezing-thawing" fixation.

In an effort to isolate the effect of freezing from the effect of subsequent dehydration in vacuo, tissues were frozen and placed in organic solutions at temperatures of from -40° to -78°C . The only solvent used at the lower temperature was Methyl Cellusolve (National Carbon Co.); Methyl Cellusolve, absolute ethyl alcohol, anhydrous diethyl ether, and anhydrous chloroform were used at the higher temperatures. This procedure will be called "freezing-substitution".

Vacuum dehydration in the freezing-drying technic was carried out at temperatures of from -30° to -50°C . After dehydration, the tissues were either removed directly to a desiccator or allowed to rise to room temperature before being transferred. Free hand sections of these tissues were examined in the manner suggested by Hoerr ('36), using 2 mm. and 3 mm. apochromatic objectives, polarized light, the cardioid dark field condenser, and vertical illumination by the Ultropak microscope.

Tissues for routine examination were denatured for at least a week in redistilled absolute alcohol. Denaturation was attempted also by heat and the use of chemical fixing agents such as osmic acid, potassium dichromate and numerous tissue fixing mixtures. These procedures appeared to have a very limited use. Embedding was done in paraffin, in nitrocellulose, or by the methyl benzoate celloidin and paraffin technic.

OBSERVATIONS

1. Effect of the size of the piece of tissue frozen

It was observed, as was noted by Hoerr ('36), that even relatively large pieces of tissue might be frozen in chilled isopentane without cracking, with a final appearance that compared favorably under the lower powers of the microscope with similar tissues preserved in such a mixture as Bouin's fluid (figs. 1 and 6). This was true if general histologic stains were used. With more complex mixtures of dyes, such as Heidenhain's modification of Mallory's connective tissue stain (the Azan stain), even low powers of the microscope revealed

striking variations in the response of different layers of the tissue to the dyes. Guinea pig liver, for example, showed three rather sharply demarcated zones of staining. The outer zone took the stain in much the same manner as a well-fixed Zenker-formol preparation. Inside this zone was another in which the cells took the azocarmine and orange G rather too strongly. Next to this was yet another zone which stained in a nearly normal fashion, possibly taking the aniline blue a little too strongly. There then appeared in succession one or more repetitions of the last two zones, the difference in staining between adjacent layers being not so marked as in the outer regions. With the regular Mallory connective tissue stain, the irregularities of staining in laminae appeared even more marked.

Examination of this material under a 2 mm. oil immersion apochromatic objective revealed that the outer "normally" stained zone described above presented a quality of fixation which compared very favorably with the best obtainable chemical fixation of guinea pig liver. The homogeneity of the nuclear juice and of the cytoplasmic background, the distinctness and uniformity of the mitochondria, the perfectly spherical fat vacuoles, the highly refractile cell membrane, the clear-cut staining of the walls of the bile capillaries, the smoothness of the connective tissue fibers, all pointed to the fact that here fixation was equal to or superior to the best chemical fixation yet devised. It may be noted that there existed on the very surface of the block a zone, usually only one cell thick, in which the cells tended to be darkly stained. This was true of all tissues and all stains tried. It appeared to be characteristic of frozen-dried material. No reason for this has been found.

The second major zone of tissue presented a marked contrast to that just described. Throughout this zone the cells showed serious artefacts of freezing. The ice crystals produced in the cytoplasm had disrupted the fat vacuoles and deposited proteins along their edges so that mitochondria were

not identifiable. The nuclei were badly reticulated by ice crystals and the connective tissues were hardly recognizable.

The next major area, laminated in staining density, was more or less uniform in quality of fixation, the quality falling off toward the center of the larger blocks. In this area the connective tissues were poorly preserved. A pattern of large ice crystal artefacts broke up the connective tissue nuclei and fibers generally. Blood vessels containing blood showed a reticulate structure in the lumen in contrast to the uniform preservation of the erythrocytes and white cells in the first described outer zone. The preservation of epithelial cells of the area represented a compromise between the excellent outer and the very poor second zones described above. The artefacts were small enough so that nuclear detail was preserved fairly well, although a definite disruption by ice crystals was uniformly apparent. Cytoplasmic detail was quite well preserved, but here too, a fine reticulation was generally present. The differences which appeared between the secondary zones within this general area indicated that preservation was poorer in the cells taking the azocarmine and orange G too strongly.

The same type of zoning occurred in other tissues where the mass was relatively great and the organ more or less compact. Guinea pig suprarenal gland, after isopentane freezing, usually showed excellent preservation of the zona glomerulosa and of the outer part of the fasciculata. The inner part of the fasciculata usually contained a narrow zone in which nuclear ice crystals were very large. The lipin droplets in this area were less regularly round and smooth; the nuclei were badly broken by ice artefacts. Deeper in the fasciculata, reticularis, and medulla the quality of fixation was again quite good, though still inferior to that in the outermost zone. That this phenomenon was not due to inherent differences between the parts of the gland was shown by the fact that wherever a little of the surface of the organ was covered by adherent connective tissue, the zone of poor fixation shifted toward the capsule and came to lie in the zona glomerulosa or even outside the organ in the connective tissue covering.

Small bits of these tissues or of others did not show all the zones described after isopentane freezing. If small enough, the fixation might be excellent throughout. No absolute limits can be stated for a maximum size in which this type of preservation occurs exclusively since the thickness of the outer zone varies over rather wide limits in different tissues. Reasons for the variation in the response of different tissues to freezing will be discussed later.

Tissues frozen by compression between the chilled brass block and a nonconductor such as paper or cork substantiated the observations recorded above. Here the heat was conducted away from one side of the tissue only and therefore freezing had perforce progressed through the entire sample from that side. The picture obtained by this method corresponded to that resulting from isopentane freezing with the exception that the general quality was lower throughout (fig. 1). From a fairly good surface zone (fig. 2), a zone (fig. 3) was delimited which was so disrupted that, were it alone, the tissue might not have been recognizable. Beyond this again appeared fixation (fig. 4) that at least permitted recognition of the tissue although it was poorly preserved in comparison with the surface layer.

2. The effect of the relation of tissue volume to surface area

Certain tissues, because of their peculiar structure, have a large surface area in relation to their volume. Such are the following guinea pig tissues: pancreas, duodenum which has been slit open, gastric mucous membrane, and, to a lesser extent, the epididymis. It is possible to make very thin slices of kidney and liver, also, and to increase artificially the surface of testis and pancreas by teasing and even by smearing them onto cover slips before freezing. When such tissues were frozen and dried, the quality of the fixation was superior to that of the same tissues frozen in compact masses. In most cases the fixation partook of the characteristics of the outer zone previously described, but in the testis the zone of the

best freezing-drying extended only a few cells from the surface (fig. 8).

Materials prepared by the "freezing-substitution" method previously described, showed a quality of fixation in most respects exactly comparable with frozen-dried tissues. The zoning of ice crystal artefacts and the very presence of these artefacts indicated that the freezing proper and not the dehydration or any subsequent step in the freezing-drying process was responsible for the characteristics of the material described above. Fixation by the "freezing-substitution" method is essentially a chemical one, however. By this method proteins are precipitated in a denatured state and react to stains in a fashion characteristic of chemically fixed tissues.

3. Effect of water content of the tissue frozen

It has been uniformly true that the tissues which are most fluid are the ones in which the most severe damage by ice crystals has been observed. Tissues in which this severe disruption has been found more than a very short distance from the surface are: loose connective tissue, nervous tissue, testis, and portions of the blood vascular system such as lymph nodes, spleen, bone marrow, and blood itself.

Attempts to reduce the amount of water in tissues by depriving the animals of food and water prior to killing (Bensley and Gersh, '33 a) have met with only partial success. The best results following this procedure have been in tissues where freezing-drying is already most successful in undehydrated animals. Little, if any, improvement has followed its use in preparation of testis, spinal cord, and brain—tissues that are the most difficult to preserve satisfactorily by freezing-drying.

4. Effect of the rate of freezing

In an attempt to discover whether there existed an optimum rate of freezing other than the fastest rate obtainable, a series of freezings was carried out at temperatures of -60°C. , -95°C. , -120°C. , -140°C. , -165°C. , -184°C. , and

—195°C., using guinea pig liver as the test tissue. The typical appearance of guinea pig liver frozen in isopentane at the lowest temperature before it solidifies has already been described. In this series of experiments it was found that the only result of the elevation of the freezing temperature could be interpreted as a gradual shifting of the zones of fixation toward the outside of the block. Thus, at the highest temperature, the fixation of the tissue as a whole was almost identical with that of the inner zone of the tissue previously described. Intermediate freezing temperatures of —100°C. to —140°C. showed preservation in which the surface freezing was comparable with that of the intermediate very poorly fixed zone of the material frozen at —195°C.

5. Effect of the temperature of dehydration

In general, tissues dehydrated at —40 °C. exhibited a more uniform quality of preservation than those dehydrated at —30°C. or above. On the other hand, for purposes of cellular morphology, at least, there did not appear to be any choice between —40°C. and —50°C. The greatly increased time necessary for dehydration at the lower temperature is an argument against its general use.

6. Effect of the rate of dehydration

At lower temperatures of dehydration, the use of a triple stage mercury diffusion pump throughout the period of dehydration was not found to cause disruption of cells by too rapid removal of water vapor as was thought to be the case (Hoerr, '36) at temperatures of —25°C. to —30°C.

7. Effect of miscellaneous factors in handling dried tissue

The rapidity of handling dried tissues was of extreme importance in preventing them from taking up atmospheric moisture. Particularly was this true of fatty tissue such as frequently surrounds the pancreas. Pieces of dried pancreas were found to take up water so rapidly from a humid atmo-

sphere that before free hand sections could be cut the entire block was soft and spongy. To avoid the further complication of atmospheric moisture condensing on cool blocks, it was desirable to allow the blocks to come to room temperature before exposing them to the air at all.

Since the freezing-drying process does not completely remove water from tissues, it was thought cells might be caused to shrink in the process of vacuum embedding. Denaturation of the tissues in redistilled absolute alcohol for a week or more left the proteins in such condition as to permit shrinkage in the process of paraffin embedding following cedarwood oil clearing. The best preservation of general histological detail followed embedding in celloidin without the use of heat. This method of preparation has, for the first time in the author's experience with frozen-dried tissues, preserved the characteristic differences between collagenous and reticular connective tissue fibers in the loose interstitial connective tissue of the epididymis. This technic has also been successful in preventing shrinkage of spermatocytes in active mitosis (fig. 8), something which had hardly been seen, except for early pro-phases, in frozen-dried testis embedded in paraffin.

DISCUSSION

Four factors have been described which are believed to modify the quality of cytological fixation by the Altmann technic. A study of these factors might be expected to throw some light on the reasons why Bensley and Gersh ('33) found that freezing was most satisfactory in large pieces of tissue and in tissues made larger and protected by an added covering of other tissue; as well as why now our best cellular preservation results from the use of the very outside of small bits of tissue having a relatively large surface area.

Bensley and Gersh ('33, p. 210) state that: "In the original experiments the cytological fixation was least satisfactory at the surface of the blocks, due to the formation of ice crystals."

Scott ('33) explained this improved fixation beneath the surface on the basis of the direct observations by Chambers and Hale ('32) of freezing of muscle fibers. These workers observed that preceding freezing there was a dehydration due to the freezing out of "free" water and the consequent elevation of the salt concentration within plasma membrane. Scott further suggested that equilibrium of temperature was probably not reached for some time after a piece of tissue was immersed into liquid air and that free water might be frozen out first in this case as it was in the much slower process described by Chambers and Hale. His basis for this contention was that the tissue was not in contact with liquid air but with air in the gaseous form, which is a rather poor conductor of heat. The superior results obtained by freezing in a liquid such as ethyl alcohol, pentane, or isopentane are easily understood if the rate of freezing is a major factor, for at ordinary temperatures these liquids conduct heat at nearly eight times the rate of nitrogen gas. Attempts to speed freezing by the use of a relatively great mass of brass, the heat conductivity of which is nearly 600 times that of ethyl alcohol, proved unfruitful because it was impossible to change the surface of the block that was in contact with the tissue. Fluid baths have the further advantage of contact with all surfaces of the tissue simultaneously.

Satisfactory preservation by freezing-drying of large pieces of tissue appeared to be limited less by the heat conductivity of the freezing medium than by that of the already frozen surface of the tissue, a factor which can hardly be controlled. So long as the fluid bath had a higher rate of conductivity than frozen tissue had, the use of different fluids for freezing media could affect the preservation only within a thin surface layer. It has been observed that the organs most satisfactorily preserved are those with a loose lobular arrangement. This may be correlated with the fact that the connective tissue partitions of these organs are bulky and probably freeze readily by the propagation of ice crystals from the surface inward. The ice thus formed is a much better conductor of heat than is liquid

water, and allows a rapid withdrawal of heat from the parenchymatous portion of the organ.

The experiments in which freezing was produced over a wide range of temperatures showed that the effect of lowering the freezing temperature did not bear a constant relation to the adequacy of the resulting fixation. These experiments indicated that freezing preservation was best when the temperature of the isopentane was as low as possible, certainly below $-160^{\circ}\text{C}.$, and that if this temperature or lower was not obtained, the next best result could be expected at such a temperature that the zone of very large ice crystals was not produced, that is, about $-60^{\circ}\text{C}.$ Similar results were at times obtained directly from liquid air or liquid nitrogen.

The zones produced within the area of "secondary" freezing cannot yet be explained satisfactorily. The morphological and staining reaction differences between adjacent zones must be interpreted as evidence of physical and chemical alterations of the tissue in the freezing process. Many factors complicate the interpretation of this area. One of these is the possibility of development of considerable pressure inside the block as the surface freezes. The expansion ordinarily accompanying the formation of ice crystals is prohibited by the surface shell of relatively inelastic frozen tissue. The resulting pressure may either crack the block, as it frequently does, or may cause a further slowing of the internal freezing through liberation of heat of compression and through depression of the freezing point by pressure, both of which are well recognized phenomena.

Since the rate of freezing of the interior of the block is determined by the rate of heat conduction by the already frozen shell of tissue, and is possibly slowed by the increased pressure, it seems probable that the quality of the preservation in this area may depend upon the freezing out of water and the concentration of salts within the cells, as was found by Chambers and Hale to be the case in the slow freezing of tissues. The fact that in most tissues the ice crystal artefacts in the intercellular spaces within the block were much larger

than the artefacts within the cells of the same region further supports this view. To be sure, a part of this may be due to original differences in water content of the cells and the intercellular spaces. The fact that the cells in this region differed so greatly in their freezing artefacts, although not greatly in water content, leads us to believe that within some cells the freezing was initiated rather soon by the appearance of an ice crystal nucleus, while in others the lack of the appearance of such a nucleus permitted the same type of diffusion of water out of the cell to the intercellular spaces as was noted in the work of Chambers and Hale. Kistler ('36) pointed out that there is adequate reason to believe that in biological systems the water is in such small capillary droplets that much of it may be cooled to very low temperatures without freezing, since as the temperature is reduced, the activity of the water decreases as does also the tendency toward appearance of nuclei of crystallization. Water in cells in which no crystal nuclei appear can freeze only by diffusion out of the cell onto an already frozen surface. The intercellular spaces, which are continuous over relatively great distances, provide such a freezing surface.

If these changes in the distribution of intracellular fluid take place within blocks of tissue, it may be expected that changes in the intracellular distribution of highly soluble and highly diffusible salts accompany them. Studies on the precise distribution of such salts, then, appear best confined to the surface zone of tissues that are rapidly frozen at the lowest possible temperature in a fluid from which gas is not evolved during the freezing process.

Concerning the effect of water content of tissues on the success with which they may be frozen-dried, it is of interest to note the amount of change in weight of animal tissues through dehydration by acute thirst, presumably mainly by loss of water. Kudo ('21) noted in a series of adult albino rats that acute thirst produced a gross weight loss of 36.1%. Tissues which have been most successfully frozen following dehydration of the animals, with the weight loss by thirst of

the same tissues from Kudo's series, are: pancreas (53.1%), liver (37%), stomach and intestines (36.4%), epididymides (30.0%) and kidney (23.8%). On the other hand, the failure of dehydration to improve the fixation of testes is explained by the fact that acute thirst causes loss of only 15.1% of their weight. Failure to freeze central nervous system tissues more satisfactorily following dehydration of the animal is correlated with the fact that in acute thirst the brain increases in weight by 0.12% and the spinal cord by 1.80% (Kudo, '21).

CONCLUSIONS

These studies on the process of freezing and on other factors controlling fixation by the Altmann method lead us to the following conclusions:

1. The nature of the coarsest reticulation in tissues prepared by the Altmann technic is due to ice crystals and not to the treatment of the tissue after freezing. This was established by (a) the larger size of the artefacts in tissues with a high water content, (b) the absence of such artefacts in tissues prepared by the freezing-thawing method, and (c) the presence of such artefacts in tissues prepared by the freezing-substitution method.

2. The degree of this reticulation is influenced by: (a) the rate of freezing, (b) the water content of the tissue originally and as reduced by dehydration of the animal prior to killing, and (c) changes that occur in free water and salt concentration during the process of freezing.

3. Preservation of cytological details in the outer layer of tissues frozen in chilled isopentane at -195°C . surpasses in excellence chemically-fixed tissue. The completeness of the cellular constituents in a form unmodified except for drying makes this the fixation of first choice for purposes of cellular chemistry.

4. The freezing fixation within larger blocks of tissue is inferior to that of the surface zone, from which it is separated by a layer of very poorly preserved tissue. This inner zone may be, nevertheless, superior to chemical fixation for general

cytological and histochemical studies. The zone is not suited to precise chemical studies since it is probable that, at least in part, the general quality of preservation is due to changes in water and salt concentrations during the freezing process.

5. Denaturation of proteins of frozen-dried tissue preparatory to morphological studies is best assured by the treatment of the blocks for at least a week in redistilled absolute ethyl alcohol. This should precede clearing and embedding for optimum histological preparations.

6. Since tissues are subject to modification by heat even after alcohol denaturation, embedding in celloidin is recommended as a routine procedure.

It is with pleasure and gratitude that I acknowledge my indebtedness to Professor R. R. Bensley for kindly criticism and helpful suggestions and to Doctor N. L. Hoerr, under whose direction this work was carried out, for continuous encouragement and cooperation. To all the members of the Department of Anatomy I am grateful for many courtesies shown me during the period of this investigation.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

1 Guinea pig liver frozen by contact with a chilled brass plate and dehydrated in vacuo. The surface to the left was against the brass; that to the right against cork at room temperature. Dried block denatured in alcohol. Paraffin embedded. Stained with Hansen's chrome-alum-hematoxylin and eosin. Photomicrograph across the width of an entire section. $\times 41$.

2 Portion of a high power field near the left edge of the section shown in figure 1. $\times 370$.

3 Portion of a field near the center of the same section. $\times 370$.

4 Portion of a field near the right edge of the same section. $\times 370$.

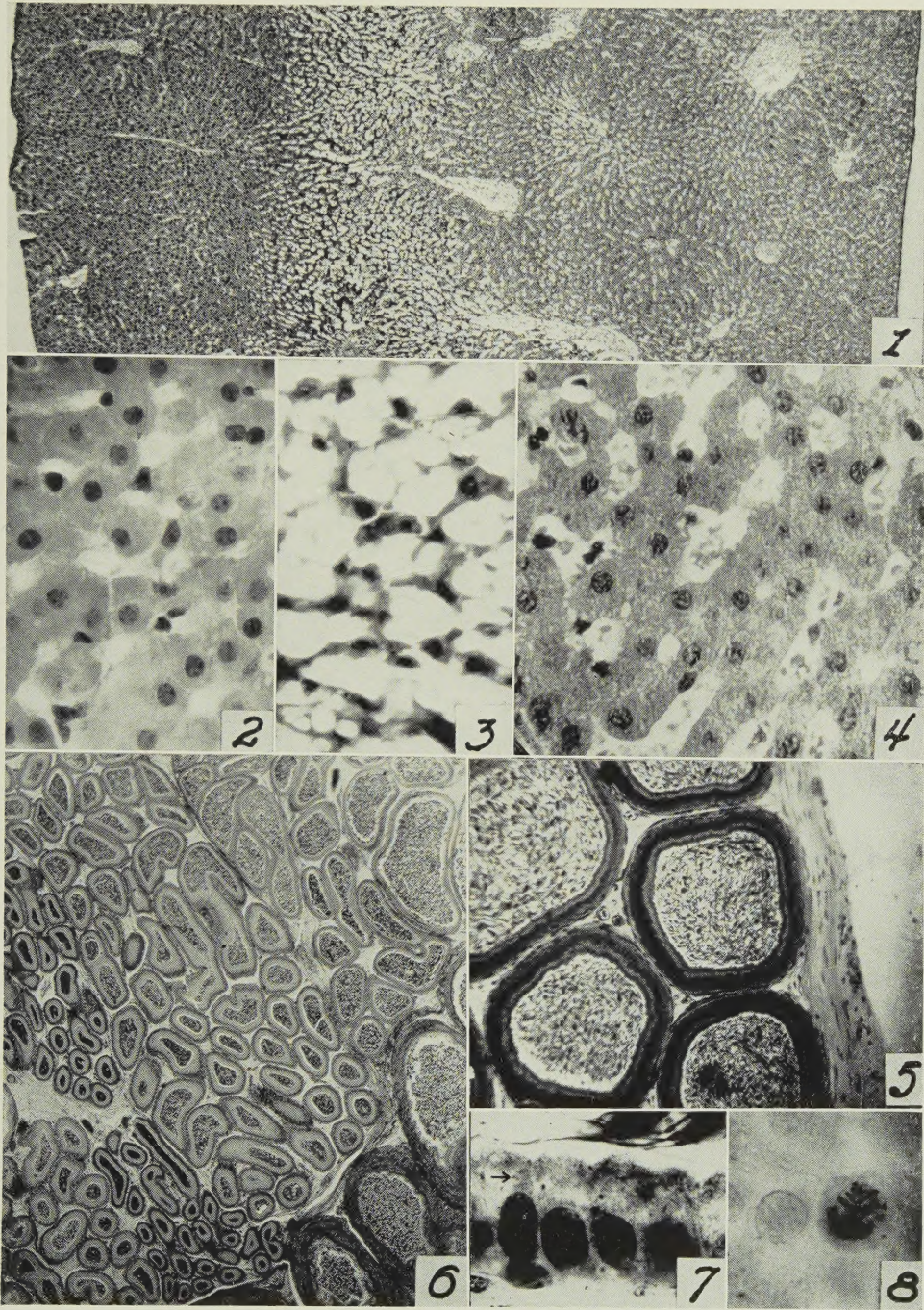
5 to 7 Guinea pig epididymis. Frozen in chilled isopentane and dried in vacuo at -40°C . Alcohol denatured. Celloidin embedded. Stained as indicated below. All are photomicrographs.

5 Stained with iron hematoxylin. This field shows the freedom from shrinkage artefacts and the general high quality of fixation of the surface region of tissues by the Altmann technic. $\times 140$.

6 Mallory-Azan stained. This very low power indicates how well relatively large pieces of loosely arranged tissues may be preserved by freezing-drying. $\times 20$.

7 Stained with iron hematoxylin. Oil immersion. Shows the quality of cellular preservation. Note central body and centrioles near end of arrow. $\times 1000$.

8 Guinea pig testis showing the preservation of a cell in mitosis near the surface of the frozen tissue. Such preservation extends at best only a few cells deep in testis. Celloidin section. Iron-hematoxylin. $\times 1000$.



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